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Transcription of lignocellulose-decomposition associated genes, enzyme activities and production of ethanol upon bioconversion of waste substrate by *Phlebia radiata*

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Abstract

Previously identified twelve plant cell-wall degradation associated genes of the white rot fungus *Phlebia radiata* were studied by RT-qPCR in semi-aerobic solid-state cultures on lignocellulose waste material, and on glucose-containing reference medium. Wood-decay involved enzyme activities and ethanol production were followed to elucidate both the degradative and fermentative processes. On the waste lignocellulose substrate, *P. radiata* CAZy genes encoding cellulolytic and hemicellulolytic activities were significantly up-regulated whereas genes involved in lignin modification displayed a more complex response. Two lignin peroxidase genes were differentially expressed on waste lignocellulose compared to glucose medium, whereas three manganese peroxidase encoding genes were less affected. On the contrary, highly significant difference was noticed for three cellulolytic genes (*cbhI_1, eg1, bgl1*) with higher expression levels on the lignocellulose substrate than on glucose. This indicates expression of the wood-attacking degradative enzyme system by the fungus also on the recycled, waste core board material. During the second week of cultivation, ethanol production increased on core board to 0.24 g/L, and extracellular activities against cellulose, xylan and lignin were detected. Sugar release from the solid lignocellulose resulted with concomitant accumulation of ethanol as fermentation product. Our findings confirm that the fungus activates its white rot decay system also on industrially processed lignocellulose adopted as growth substrate, and under semi-aerobic cultivation conditions. Thus, *P. radiata* is a good candidate for lignocellulose-based renewable biotechnology to make bio-fuels and bio-compounds from materials with less value for recycling or manufacturing.

Keywords: white rot fungi, wood decay, bioethanol, lignocellulose biodegradation, bioconversion, gene expression

Introduction

Basidiomycota white rot fungi are able to degrade all the components of renewable lignocellulosic biomasses like wood, and are thus promising candidates for biotechnological applications such as pre-treatment of feedstock plant materials for conversion to bioethanol, other biofuels, and production of value-added bio-products. Wood-decay enzyme encoding genes of *Basidiomycota Agaricomycetes* fungi have been studied widely. Large genome sequencing projects and comparative studies have revealed differences in the gene content of fungal genomes and have aided in prediction of fungal life-styles and biomass degradation abilities (Eastwood et al. 2011; Floudas et al. 2012; Ruiz-Dueñas et al. 2013; Lundell et al. 2014; Nagy et al. 2017).

Subsequent genome wide transcriptome and proteome analyses have enabled identifying the functions of annotated genes involved in wood decay and plant biomass degradation, especially the CAZyme encoding genes (Martinez et al. 2009; Vanden Wymelenberg et al. 2009; Hori et al. 2014; Kuuskeri et al. 2016). In addition to these elaborate and holistic omics studies, transcriptional analyses of specific sets of lignocellulose-degradation associated genes at defined growth and nutritional conditions have been conducted for a few white rot fungal species revealing both substrate- and time-dependent regulation of gene expression (MacDonald and Master 2012; MacDonald et al. 2012; Rytioja et al. 2014a; Skyba et al. 2016).

Enzymes acting on plant cell wall carbohydrate polymers are classified in the Carbohydrate-Active enzyme database (CAZy, www.cazy.org, Lombard et al. 2014). Synergistic action of different enzymes are needed for decomposition of the polysaccharides cellulose and hemicelluloses (Lundell et al. 2014; Rytioja et al. 2014b). Fungal enzymes necessary for decomposition of cellulose microfibrils include endo-β-1,4-glucanases, exo-β-1,4-glucan cellobiohydrolases and β-glucosidases (van den Brink and de Vries 2011; Rytioja et al. 2014b). Lytic polysaccharide monooxygenases (LPMOs, CAZy class AA9) are a novel group of extracellular enzymes attacking cellulose chains via an oxidative mechanism (Horn et al. 2012). Several CAZy enzymes with different specificities, such as endo-β-1,4-xylanases, β-1,4-xylosidases, β-mannosidases, acetyl xylan esterases, α-arabinofuranosidases and α-glucuronidases are needed for the hydrolysis of hemicelluloses depending on their chemical structure and side-chains present (van den Brink and de Vries 2011; Lundell et al. 2014; Rytioja et al. 2014b).

For utilization of cellulose and other polysaccharides, the compact plant biomass (i.e. lignocellulose) is structurally opened by chemical or physical pre-treatment methods that are generally used in e.g. biorefineries to enhance enzyme treatment and to convert or release lignin units (Gillet et al. 2017). It is assumed that oxidation and depolymerization of lignin present a bottleneck for the industrial usage of lignocelluloses of various sources (such as wood and grass plants). However, lignin can be modified and even mineralized by white rot fungal extracellular oxidoreductases, including lignin and manganese peroxidases (Hofrichter et al. 2010; Hatakka and Hammel 2011), in association with hydrogen peroxide generating enzymes such as glyoxal oxidases or aryl alcohol oxidases (Hammel and Cullen 2008; Martínez et al. 2009; Lundell et al. 2010, 2014; Floudas et al. 2012). In addition, for example laccases are believed to be involved in modification of lignin structures through aromatic mediators although the role of these enzymes in degradative processes is not evident (Lundell et al. 2010).

The taxonomic type species of the genus *Phlebia*, *Phlebia radiata*, is a white rot fungus classified to the phylum *Basidiomycota*, class *Agaricomycetes*, order *Polyporales*, and phlebioid clade (Kuuskeri et al. 2015). As a saprotroph and white rot fungus, *P. radiata* is able to colonize wood and degrade all the main components of lignocellulose (Nakasone and Sytsma 1993; Hakala et al. 2004; Kuuskeri et al. 2015, 2016). Genome sequencing uncovered the overall CAZy gene content of *P. radiata* genome. Proteomic and

transcriptional analyses of the fungus growing on solid spruce (*Picea abies*) wood revealed expression of activities against cellulose, hemicellulose, lignin and pectin (Kuuskeri et al. 2016). Especially lignin-attacking class-II peroxidase encoding genes (*lip* and *mnp* genes) were found to be highly up-regulated at the earlier time point of spruce wood cultivation indicating functions for these oxidoreductases as primary attackers on wood lignin (Kuuskeri et al. 2016). Few of the lignin-modifying enzyme encoding genes of *P. radiata* have been characterized in previous studies (Saloheimo et al. 1991; Hildén et al. 2005, 2006; Mäkelä et al. 2006) and their corresponding enzymes demonstrate potential as redox biocatalysts on wood lignin and aromatic compounds (Hofrichter et al. 2001; Lundell et al. 2016). The polysaccharide-active CAZy genes have so far been of less concern. Recently, the lignocellulose converting efficiency of the fungus *P. radiata* was extended to production of ethanol from non-pretreated wood-fiber based lignocellulose waste material in a single-step process under semi-aerobic cultivation conditions (Mattila et al. 2017). Of the several phlebioid species studied, *P. radiata* was the most efficient in ethanol production thus making the fungus a promising candidate for waste-lignocellulose bioconversion processes and forthcoming applications.

The main aim of our current study was thereby to investigate the expression of lignocellulose-degradation associated genes and production of corresponding enzyme activities while *P. radiata* was cultivated on waste core board material and undergoing fermentation metabolism to produce ethanol. Data from the genome-wide transcriptional and proteomic analyses of *P. radiata* cultivated on solid spruce wood (Kuuskeri et al. 2016) was used for selection of twelve highly up-regulated CAZy genes for RT-qPCR analysis. Solid spruce wood cultivations were considered to be close to the natural habitat conditions of the fungus while the recycled waste lignocellulose (core board) substrate and semi-aerobic cultivation conditions were less usual or natural-like, and thereby, presenting an interesting comparison discussed in this study.

Materials and methods

Fungal cultivations

P. radiata isolate 79 (FBCC0043) was obtained from the HAMBI Fungal Biotechnology Culture Collection of the University of Helsinki (HAMBI-FBCC, fbcc@helsinki.fi). Identification of the isolate has been confirmed by ITS-PCR (Kuuskeri et al. 2015; Mali et al. 2017), with nuclear and mitochondrial genomes sequenced (Salavirta et al. 2014; Kuuskeri et al. 2016). The isolate was maintained on 2 % (w/v) malt extract agar (2 % w/v) at 25 °C in the dark throughout the study.

Cultivations were performed in 100 ml glass Erlenmeyer flasks under semi-aerobic conditions essentially as described previously (Mattila et al. 2017). The phosphate-buffered yeast-extract containing

liquid medium, pH 6 (Okamoto et al. 2010), was adopted in 20 ml volume in each flask. Carbon source of the medium was either 1.0 g dry weight of core board solid lignocellulose or 1 % (0.2 g/20 ml) glucose. Core board is an abundant wood-fiber based municipal waste material with no other feedstock applications for manufacturing or further potential for recycling. The material contains 73.1 % carbohydrates as polysaccharides and 14.2 % lignin (of dry weight; analyzed by Labtium Oy, Espoo, Finland). Approximately 58 % glucose, 10 % xylose and 4.1 % mannose are the main sugar units of the core board polysaccharides. The color of the material is light grey-brown without any signs of ink or dye compounds. According to previous HPLC analyses (Mattila et al. 2017), no dissolved aromatic or phenolic compounds are released to water phase from ground core board during the cultivations. To our knowledge, no harmful adhesives or other potentially (fungal growth) inhibiting compounds are used in manufacturing of core board. Four biological replicate cultures of each carbon source and time point were cultivated at 25 °C in the dark for six weeks. Culture flasks were closed with tight rubber stoppers (Mattila et al. 2017). Culture supernatant samples were collected every week. The solid material (lignocellulose substrate including fungal mycelia) of each flask was collected at time points 2, 4 and 6 weeks of cultivation, quickly frozen with liquid nitrogen, and stored at - 80 °C.

Enzyme activities, ethanol assay and sugar release measurements

Laccase, manganese peroxidase, xylanase, endoglucanase, cellobiohydrolase and β-glucosidase activities were measured from the culture supernatants adopting previously described assay methods (Rytioja et al. 2014a; Kuuskeri et al. 2015). Ethanol concentration of the culture supernatants from time points 1, 2 and 3 weeks were measured by using a K-ETOH spectrophotometric-detection based assay (Megazyme, Bray, Ireland) as described previously (Mattila et al. 2017) according to the instructions of the manufacturer (https://secure.megazyme.com/Ethanol-Assay-Kit). The assay was tested with generally used liquid media, including malt extract broth and the basal medium adopted in this study, and their additional components (1 % yeast extract, 1 % glucose) in order to ensure suitability of the method for the samples analyzed. Concentration of dissolved reducing sugars was determined from the culture fluid samples based on the method of reduction of dinitrosalicylic acid (DNS) (Miller 1959). All the measurements were performed using 96-well plates and Infinite M200 microplate reader spectrophotometer (Tecan, Männedorf, Switzerland) except for the cellobiohydrolase measurements which were performed using a fluorescence reader (Victor^3, PerkinElmer, Massachusetts, USA).

RNA extraction and cDNA synthesis

RNA was isolated from the frozen samples of four biological replicates from the 2 week and 4 week time points of the core board and glucose cultivations. The core board solid-state mycelial culture samples, and mycelia from glucose cultures were ground under liquid nitrogen with A11 Basic analytical mill (IKA,

Staufen im Breisgau, Germany), and mortar and pestle, respectively. Total RNA was extracted by CsCl gradient centrifugation method (Patyshakuliyeva et al. 2014). RNA was further purified by NucleoSpin RNA Clean-up kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. Concentration, purity and integrity of the total RNA were analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) using the RNA6000 Nano Assay and NanoDrop1000 Spectrophotometer (Thermo Scientific, Waltham, USA). cDNA was synthesized from 1 µg of total RNA using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) with integrated removal of genomic DNA contamination according to the instructions of the manufacturer.

Real-time quantitative reverse transcription PCR (RT-qPCR)

Data from the proteomic and transcriptional analyses of *P. radiata* grown on solid spruce wood (Kuuskeri et al. 2016) were used for selecting highly up-regulated (> 4-fold difference in at least one of the time points studied) lignocellulose-degradation associated genes for RT-qPCR analysis (Table 1). Gene models of the selected genes were manually curated according to the RNA-seq reads mapped against the genome assembly (Kuuskeri et al. 2016). Predicted amino acid sequences of the transcripts were functionally annotated according to protein Blast search homologies with known protein coding genes from other fungi.

Relative expression of the studied genes was determined with RT-qPCR from the 2 week and 4 week time points of the core board and glucose cultivations. Three technical replicates of each of the three biological replicate cultures were amplified and analyzed. Studied genes and their transcript-specific qPCR primers are listed in Table 2. Primers were designed according to genome sequencing data of *P. radiata* using PerlPrimer software version 1.1.21 (Marshall 2004). For a few genes, primers had been designed in previous studies (Table 2). When possible, the forward or reverse primer of the qPCR primer pair was designed to overlap an exon-intron junction to prevent annealing to and amplification of possible genomic DNA present in the cDNA samples.

RT-qPCR analyses were performed in 96-well white PCR plates (Bio-Rad, Helsinki, Finland) and CFX96 Real Time PCR equipment (BioRad, Helsinki, Finland) as instructed by the manufacturer. The 25 µl reaction mix contained Maxima SYBR green qPCR master mix (Thermo Scientific, Waltham, USA), 3 µM of each primer and 5 µl of the cDNA template diluted 1/10 – 1/100. Thermal cycling conditions included an initial denaturation at 95 °C for 10 min followed by 39 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds. For the melting curve analysis, temperature was increased in a stepwise fashion from 65 °C to 95 °C by 0.5 °C increment in every 5 seconds.

RT-qPCR data analysis

Amplification efficiency and specificity of the primers were assessed according to a standard curve of dilution series with a mixture of cDNA samples as a template. Amplification efficiency (E) was calculated by the CFX Manager 3.1 software (BioRad, Helsinki, Finland) according to the equation $E = [(10^{-1/slope}) - 1] * 100$ %. Amplification efficiency should be in the limits of 90-110 % (Maxima SYBR Green qPCR Master Mix manual, Thermo Scientific, Waltham, USA). Efficiency > 110 % is an indication of formation of non-specific PCR products while low efficiency may be due to PCR inhibitors, suboptimal PCR conditions or failure in primer design. Specificity of the primers and absence of genomic or external DNA was confirmed by the presence of only one qPCR product main peak in the melting curve analysis, and by adding control reactions excluding cDNA template (no-template controls) and including starting RNA but without generated cDNA (no-reverse-transcriptase controls) to the analyses.

Cq (quantitation cycle) values of the transcript amplicons were normalized with the geometric mean of the Cq values obtained for the three endogenous *P. radiata* reference gene transcripts. Reference genes were chosen according to stable expression detected in our previous transcriptome study (Kuuskeri et al. 2016). These genes putatively encode a candidate mitochondrial membrane fission protein (FIS1), a candidate ubiquitin conjugating enzyme (UBC6), and a candidate GTP-binding protein (SAR1). After normalization, relative fold change of gene expression on core board in reference to glucose cultures was calculated by the $2^{-\Delta\Delta Cq}$ method. Statistically significant changes between the two cultivation conditions or between the cultivation time points were detected by Student´s *t*-test using two-tailed distribution and two-sample unequal variance. *P* value < 0.01 was considered to indicate a significant difference. NormFinder Excel Add-In (Andersen et al. 2004) was used to assess the stability of expression (amount of transcripts under various conditions) of the reference genes after transforming their Cq-values from glucose and core board 2 and 4 week cultures to a linear scale by 2^{-Cq} conversion.

Results

Manual curation of the gene models

Mapping of the RNA-sequencing reads against the genome assembly of *P. radiata* isolate 79 and functional annotation of the predicted gene models was performed as recently explained (Kuuskeri et al. 2016). According to the initial functional annotation, the genome encodes at least one GH11 endo-β-1,4-xylanase, eight GH3 β-glucosidases, four GH5_5 endo-β-1,4-glucanases, six GH7 cellobiohydrolases, 12 AA9 lytic polysaccharide monooxygenases, five laccases (of AA1 class), six manganese peroxidases and four lignin peroxidases (of AA2 class) and at least 19 family AA3 (GMC protein superfamily) oxidoreductases including several putative alcohol oxidases (Kuuskeri et al. 2016). Gene models chosen for RT-qPCR analysis were manually curated and characterized. The gene set was designed to include

hydrolytic and oxidative activities against cellulose, hemicelluloses and lignin. Specific genes were chosen based on the highest fold change on spruce wood cultures in reference to standard malt extract medium conditions and/or the highest count value detected among the CAZy class and family (Table 1) (Kuuskeri et al. 2016). Among the selected genes, the predicted protein products of the cellobiohydrolase, endo-glucanase, β-glucosidase, endo-xylanase, and lytic polysaccharide monooxygenase all included one carbohydrate-binding CBM1 domain fused in the open reading frame of the gene. The CBM1 domain was located either in the N-terminal (EG1, BGL1) or C-terminal (CBHI_1, LPMO1, XYN1) of the protein model. The studied CAZy genes, except for the *aox1* gene, included N-terminal signal sequences, thus indicating secretion of the encoded proteins. Table 3 shows the characteristics of the studied *P. radiata* genes and their known or predicted protein products.

Gene transcription on core board waste lignocellulose

Relative expression of the selected genes was normalized against transcript abundances of three *P. radiata* reference genes: *sar1*, *ubc6* and *fis1*. According to NormFinder analysis, expression of *sar1* was the most constant (stability value 0.122) under the studied conditions and expression of *fis1* and *ubc6* was close to each other (stability values 0.213 and 0.236, respectively).

Differential regulation of target genes on core board versus glucose medium

All the cellulolytic and hemicellulolytic target genes were up-regulated ($P < 0.01$) in at least one of the time points on lignocellulose core board substrate in comparison to glucose medium (Fig. 1). The lytic polysaccharide monooxygenase encoding gene (*lpmo1*) had the highest fold change (> 360-fold after 2 weeks, > 100-fold after 4 weeks, $P < 0.01$) followed by the GH11 endo-β-1,4-xylanase encoding gene (*xyn1*) (> 90-fold after 2 weeks, > 70-fold after 4 weeks, $P < 0.01$). Of the lignin-modifying oxidoreductases, lignin peroxidase LiP2 and LiP3 encoding genes were down-regulated after two weeks on core board lignocellulose whereas the same genes were up-regulated later, after four weeks of cultivation (Fig. 1). On the contrary to the *lip* genes, none of the selected manganese peroxidase genes, encoding both long- and short-MnP enzymes (MnP1-long; MnP2-long; MnP3-short) demonstrated statistically significant changes of expression on core board as compared to the glucose cultivations. The AA3_3 GMC superfamily gene (*aox1*) encoding a putative alcohol oxidase, was in turn down-regulated at the two week time point of cultivation (Fig. 1). The *P. radiata* laccase 1 encoding gene (*lacc1*) was included in the RT-qPCR analysis although its expression was not up-regulated previously on spruce wood (Table 1). In this study, the *lacc1* gene was seemingly up-regulated after 2 weeks on core board (Fig. 1) but the relative expression levels were very low at both time points (2 and 4 weeks) and on both media (Fig. 2, Fig. 3) (Cq values were close to the threshold value 30 on core board, and just over 30 on glucose medium).

Time-dependent gene expression

Expression of *aox1*, *eg1* and *lip2* genes was significantly enhanced ($P < 0.01$, *t*-test) after the fourth week of cultivation on core board substrate as compared to the transcript levels detected from the week 2 samples (Fig. 2). On the contrary, the *bgl1*, *mnp2-long* and *mnp3-short* genes demonstrated significantly higher expression levels at the earlier time point (week 2) (Fig. 2). On glucose medium, however, only *lip2*, *lip3* and *mnp2-long* genes were expressed at significantly higher level after the second week of cultivation ($P < 0.01$, *t*-test) (Fig. 3). It is noteworthy that time-dependent expression of *lip2* gene on core board demonstrated an opposite pattern than on glucose medium (earlier high relative expression on glucose medium, later high relative expression on core board lignocellulose) (Fig. 2, Fig. 3).

Differences in expression of the studied genes on core board versus spruce wood

The studied genes were chosen for RT-qPCR analysis based on strongly induced expression on solid spruce wood according to our previous study (Kuuskeri et al. 2016). However, many differences of transcription patterns and regulation of gene expression may be detected when the transcriptome analyses on spruce wood and core board lignocellulose cultivations are compared (Table 1, Supplemental Fig. S1).

The cellulolytic GH5 endoglucanase encoding gene (*eg1*) was expressed more time-dependently on core board than on spruce wood with a significantly higher relative expression level at the later state of growth, after four weeks of cultivation (Table 1, Supplemental Fig. S1) (Kuuskeri et al. 2016). The other cellulolytic gene investigated, GH7 cellobiohydrolase encoding gene (*cbhI_1*), was in turn significantly up-regulated earlier on core board as compared to glucose medium, whereas the same gene was highly transcribed and up-regulated at both time points (weeks 2 and 4) on spruce wood (Table 1, Supplemental Fig. S1). However, with a less strict threshold for statistical significance ($P < 0.05$, *t*-test) this gene would be considered to be up-regulated also after four weeks on core board. The third cellulolytic gene encoding a GH3 β-glucosidase (*bgl1*) had similar expression patterns on core board and on spruce wood with higher expression level after the second week of cultivation on both substrates.

On spruce wood cultures, the *lip2* gene was highly up-regulated at both time points (Kuuskeri et al. 2016) with higher expression levels detected from week 2 samples (Table 1, Supplemental Fig. S1). On core board as substrate, this specific *lip* gene was likewise up-regulated but later, after 4 weeks. Similarly, *P. radiata lip3* gene was also up-regulated at week 4 time point on core board whereas on spruce wood, expression of this gene was up-regulated at both time points with higher expression levels detected from week 2 samples (Table 1, Supplemental Fig. S1). All of the three studied MnP encoding genes (*mnp1-long*, *mnp2-long* and *mnp3-short*) were up-regulated at both time points (weeks 2 and 4) on spruce wood whereas a different pattern was observed on core board. In our current study, the *mnp2-long* gene had a significantly higher expression level at week 2 time point on core board substrate as compared with week 4 (Fig. 2) whereas there was no statistical significance for time-dependent regulation detected for this

gene on spruce wood (Kuuskeri et al. 2016). On the contrary, the *mnp1-long* gene was time-dependently regulated (significantly higher expression level at week 2 time point as compared with week 4) only on spruce wood (Table 1, Kuuskeri et al. 2016). The *mnp3-short* gene in turn displayed similar time-dependent regulation pattern on core board as previously observed on spruce wood (Supplemental Fig. S1). The *lacc1* gene was up-regulated on core board after 2 weeks of cultivation but not on spruce wood whereas the *aox1* gene was highly up-regulated on spruce wood (Supplemental Fig. S1) but, in reference to the glucose medium, it was down-regulated on core board (Fig. 1).

Production of ethanol and enzyme activities

Extracellular enzyme activities and ethanol concentration were measured from the liquid phase of the lignocellulose core board cultures, and from the glucose reference medium cultures. Mycelial growth was very different on the glucose-reference medium as compared to the core board substrate cultures. The fungus forms a loose and floating mycelium in non-agitated liquid medium cultures, whereas in the lignocellulose solid-state cultures, the hyphae grow into the lignocellulose particles and thus, are mainly invisible to visual inspection. Ethanol production peaked on the glucose medium during the first week of cultivation whereas on core board lignocellulose, ethanol production was the highest at week 2 time point after which extracellular ethanol concentration dropped (Fig. 4a). During these changes, concentration of dissolved reducing sugars in the glucose-reference medium cultures dropped sharply from the initial value (10.0 g/l) to the level of 0.19 g/l already during the first week of cultivation (Fig. 4b), which is in accordance to the simultaneous high production of extracellular ethanol up to 0.62 g/l (Fig. 4a). On core board, steady content of released reducing sugars were detected up to six weeks of cultivation (Fig. 4b) indicating intake of the free sugars and further fermentation to ethanol (Fig. 4a).

On core board substrate, manganese peroxidase activities appeared during the second week of cultivation, and reached the maximal value of 27 µkat/liter at 3 week time point, after which a slow decrease of MnP activity was detected until the end of cultivation (week 6) (Fig. 5a). On glucose medium, however, only about half of the MnP activity levels were achieved. Similar to the MnP activity profile observed on core board, extracellular laccase activity increased until the third cultivation week to the maximal value of 134 µkat/liter (Fig. 5b). Noticeable is the persistence of the high laccase activity level until the end of cultivation on core board – especially when compared to the negligible (almost 0) activity values obtained on glucose medium.

For the cellulolytic and hemicellulolytic enzyme activities, similar promotive production patterns were detected on core board as were observed for the lignin-modifying oxidoreductases. Maximal activity levels of endoglucanase (15 µkat/liter), β-glucosidase (23 µkat/liter) and cellobiohydrolase (0.48 µkat/liter) (Fig. 6b-d) activities were reached during the second week of cultivation, after which the activities steadily dropped or demonstrated a changing pattern. On glucose medium, only negligible or

very low activities for these enzymes were measured. Xylanase (Fig. 6a) and cellobiohydrolase (Fig. 6d) activities, however, demonstrated repeating phases of enzyme activity increase and decrease over time. Cellobiohydrolase activity reached the highest level during the second week on core board, peaking also at week four (0.25 µkat/liter) and six (0.40 µkat/liter) time points (Fig. 6d). Extracellular xylanase activity on core board in turn increased until the third week, then dropped during week four but increased again reaching the maximal value of 49 µkat/liter during week five (Fig. 6a).

Discussion

In this study, expression of genes encoding specific lignocellulose-decomposing enzymes – both CAZymes and lignin-modifying oxidoreductases – was followed by RT-qPCR (reverse-transcriptase quantitative-real time PCR) when the ethanol-producing and efficiently wood-colonizing white rot fungus *P. radiata* (Kuuskeri et al. 2015, 2016; Mattila et al. 2017) was cultivated on waste lignocellulose core board as substrate. For comparison, the fungus was also cultivated on glucose medium. Three novel *P. radiata* genes (*sar1*, *fis1* and *ubc6*) coding for a candidate GTP-binding protein, a candidate mitochondrial membrane fission protein, and a candidate ubiquitin conjugating enzyme (UBC6) were adopted as references for analysis of relative gene expression according to steady transcript levels detected on spruce wood and malt extract medium (Kuuskeri et al. 2016). Stability of the reference genes in the current RT-qPCR analyses was as well tested. Homologous gene transcripts have been demonstrated to suit as references in studies of gene expression in other filamentous fungi of *Dikarya* (Steiger et al. 2010; Llanos et al. 2015; Castanera et al. 2015).

The lignocellulose-decomposition associated genes of *P. radiata* under study were chosen on the basis of their strong responses at transcriptional and protein-production levels indicating for induced expression on the solid spruce wood substrate (Kuuskeri et al. 2016). According to our current study, however, there apparently were distinct gene regulation processes on-going on the core board lignocellulose cultures than what was expected from the spruce wood cultivation. This may be due to chemical and structural differences of the lignocellulose substrates adopted as well as due to different cultivation atmospheric conditions and respirative (on spruce wood) versus fermentative metabolism (on core board). Other noticeable differences between the cultivation conditions were the addition of low amount of yeast extract (1 % w/v) as nitrogen source into the core board cultures, and cultivation dimensions (this study: 1 g d/w core board, 20 ml of liquid medium in 100 ml flasks; previously: 2 g d/w spruce wood sticks on 100 ml 1 % w/v agar made in water; Kuuskeri et al. 2016). Mycelial growth rate comparison between core board and spruce wood cultivations were not possible because fungal hyphae tend to grow into and colonize the solid lignocellulose substrate particles. However, the ability of *P.*

radiata to utilize core board lignocellulose as growth substrate was tested in our previous study (Mattila et al. 2017), which indicated that there are no apparent toxic or inhibitory compounds in this material to disturb fungal growth and metabolism.

It was noticed that on spruce wood, lignin peroxidase and manganese peroxidase encoding genes of *P. radiata* demonstrated the highest transcript quantities especially at the two week time point of cultivation (Kuuskeri et al. 2016). In this study on core board substrate, however, the lytic polysaccharide monooxygenase (*lpmo1*) and GH11 xylanase (*xyn1*) encoding genes were the most highly up-regulated transcripts. Copper-dependent lytic polysaccharide monooxygenases (LPMOs), classified as AA9 enzymes in the CAZy catalogue (Lombard et al. 2014), catalyze oxidative cleavage of glycosidic bonds in polysaccharides via mechanisms involving molecular oxygen and an external electron donor (Vaaje-Kolstad et al. 2010; Langston et al. 2011; Beeson et al. 2015; Courtade et al. 2016). LPMOs may act on a range of polysaccharide-backbone substrates including cellulose and hemicellulose, as well as chitin and starch (Agger et al. 2014; Vu et al. 2014). LPMOs have been widely studied recently, and they are considered as key enzymes needed for decomposition of plant biomasses (Hemsworth et al. 2015; Courtade et al. 2016). For example, when the white rot *Polyporales* phlebioid species *Phanerochaete carnosa* was cultivated on different wood sources including lodgepole pine, white spruce, balsam fir and sugar maple, an AA9 LPMO encoding gene and a GH10 xylanase encoding gene were most abundantly expressed of the cellulase and hemicellulase genes (MacDonald et al. 2011). Our current study further supports the importance of the lytic polysaccharide monooxygenases in fungal enzyme-driven decomposition processes against plant cell wall lignocelluloses.

In comparison to our previous study on the *P. radiata* transcriptome, the most notable difference was the obvious down-regulation on core board substrate of the AA3 family alcohol oxidase gene (*aox1*), which was highly up-regulated and also detected as protein in the spruce wood-decay transcriptome and total proteome of the fungus, respectively (Kuuskeri et al. 2016). The *P. radiata* gene model minus.g11349, however, revealed no evidence for N-terminal signal sequence indicating that the protein product is not secreted. The translated protein model demonstrates 89 % amino-acid sequence identity with the intracellular or membrane-associated alcohol oxidase (Protein sequence accession: CDG66232) of *Phanerochaete chrysosporium*, which is able to catalyze oxidation of primary and secondary alcohols, and glycerol (Shary et al. 2008; Linke et al. 2014). *P. radiata aox1* gene has demonstrated an obvious time-dependent pattern of expression since a significantly higher level of expression was detected from the second week samples of the spruce wood cultivations (Kuuskeri et al. 2016). If *P. radiata* AOX1 is associated with extra-hyphal membranes, as has been suggested for the *P. chrysosporium* homologue (Shary et al. 2008), the purpose of the enzyme could be protection against stress caused by aerobic environment, or even production of H_2O_2 for the lignin-modifying peroxidases. Therefore, the noticeable

down-regulation of the *aox1* gene on core board lignocellulose could be explained by repression caused by the oxygen-depleted, fermentative semi-aerobic atmosphere conditions in the culture flasks.

The time points of weeks two and four were selected for the RT-qPCR analysis because they correspond to the time points selected for the *P. radiata* transcriptome analysis on spruce wood (Kuuskeri et al. 2016). It is possible that expression of for example the MnP encoding genes may have been up-regulated during the first week of cultivation on core board. However, the ethanol-fermentative semi-aerobic to anaerobic atmospheric conditions may have affected as principal environmental regulative effectors for metabolic activities and expression of lignin-modifying and other oxidoreductase enzyme encoding genes. In addition, the carbohydrates are apparently more easily available for the fungus from the ground core board substrate than from solid spruce wood, which may explain the low expression of especially manganese peroxidase encoding genes on core board.

Substrate and time-dependent changes in expression of specific genes encoding lignocellulose-decomposing activities have been detected in previous studies with other white rot *Polyporales* fungi (Broda et al. 1995; Janse et al. 1998; Stewart and Cullen 1999; MacDonald and Master 2012; Korripally et al. 2015). For instance, transcription patterns of cellobiohydrolase (CBH) encoding genes of CAZy families GH6 and GH7 indicate both time-dependent regulation and dependence on growth substrate (Vallim et al. 1998; Rytioja et al. 2014a). Of the oxidoreductases, the fungal MnP encoding genes display differential regulation in several species of white rot fungi depending on e.g. nutritional nitrogen source and presence of metal ions (Gettemy et al. 1998; Johansson et al. 2002; Cohen et al. 2002; Hildén et al. 2005; Mancilla et al. 2010; Marinović et al. 2017). In general, differential regulation of genes of the same large gene family has been explained to be beneficial for adaptation of the fungus to changing environmental conditions.

Considering the extracellular enzyme activities detected, core board as substrate stimulated production of cellulolytic and xylanolytic activities, as well as expression of the corresponding target genes (*cbhI_1*, *bgl1*, *eg1*, *xyn1*). Cellobiohydrolase (CBH) and β-glucosidase (BGL) activity patterns in *P. radiata* core board cultures were quite different in comparison to the previous solid-state spruce wood cultivations, in which the CBH activity appeared after three weeks of cultivation whereas the BGL activity stayed at constant levels throughout the six-week cultivation period (Kuuskeri et al. 2016). Production of xylanase activity by *P. radiata* on core board in turn displayed similar oscillating trend as was previously observed on spruce wood (Kuuskeri et al. 2016). Overall, enzyme activities extracted from the solid spruce wood cultivation were lower than corresponding activities obtained on core board substrate and measured from the medium liquid phase. This difference is partially due to a dilution effect caused by protein extraction from spruce wood substrate (from 2 g to 40 ml of extraction buffer) (Kuuskeri et al. 2016). In accordance to our findings, the white rot *Polyporales* species *Dichomitus squalens* produced cellulolytic activities throughout the cultivation on crystalline cellulose as growth substrate (Rytioja et al.

2014a), with xylanase and endoglucanase activities increasing during the first three cultivation weeks whereas BGL activity reached the maximum earlier.

Regarding lignin-modifying oxidoreductases, laccase and MnP activities reached the highest levels relatively late, after two weeks on the core board substrate, whereas on spruce wood, these activities peaked already during the first week of growth (Kuuskeri et al. 2016). This indicates that an earlier genetic response for expression of activities against lignin is needed in the presence of intact wood than on the more processed industrial fiber-lignocellulose substrate like core board. These results imply that on the lignocellulose waste core board substrate, the initial fungal oxidative burst and enzymatic attack against lignin that was observed on spruce wood as substrate (Kuuskeri et al. 2016) may not be needed. In woody plant biomass and lignified plant cell walls, the lignocellulose polysaccharides are in a composite and ordered form and glued together by lignin moieties (Boerjan et al. 2003). Thereby, it may be emphasized that the wood carbohydrates and nutrients are apparently more easily available in the heavily processed and recycled wood-fiber based core board material than in intact, solid wood.

The waste lignocellulose core board material is known to contain fermentable glucose and xylose sugar units (Mattila et al. 2017, and this study). Immediate availability and active intake of the sugar carbohydrates is supported by a decrease in the amount of reducing sugars after the second cultivation week on core board – when production of ethanol ceased – indicating active decomposition of the substrate polysaccharides by the fungus. Accumulation of extracellular ethanol in the second week of cultivation of *P. radiata* on core board lignocellulose was in accordance with our previous study on the same substrate under similar and further optimized ethanol production conditions resulting with ethanol yields of 0.4-5.9 g/l (Mattila et al. 2017). The following quick drop in ethanol production after two weeks may be suggested to be a consequence of further metabolism of secreted ethanol. With another representative of the genus *Phlebia*, isolate *Phlebia* sp. MG-60, ethanol production from pre-treated oak wood substrate under semi-aerobic conditions occurred together with extracellular cellulose degradation and production of xylanase activities (Kamei et al. 2012a). Cultivation of the fungus on unbleached hardwood kraft pulp and waste newspaper yielded 8.4 g/l and 4.2 g/l ethanol, respectively (Kamei et al. 2012b), which is at similar levels as obtained in our previous study with *P. radiata* (Mattila et al. 2017).

On the contrary to core board lignocellulose or solid spruce wood (Kuuskeri et al. 2016) as growth substrates, RT-qPCR and enzyme activity results from the reference medium with glucose but without lignocellulose supplementation indicated possible glucose-promoted repression of expression of the laccase, xylanase, endoglucanase, cellobiohydrolase and β-glucosidase encoding genes of *P. radiata*. Moreover, also the *lpmo1* gene demonstrated lower transcript expression levels on glucose medium than on core board, which may also indicate glucose repression. In line with our results, repression of cellulolytic enzyme expression by culture medium glucose has been accordingly reported for other

449 *Polyporales* phlebioid species, that is *Phlebiopsis gigantea* (Niranjane et al. 2007) and *P. chrysosporium*
450 (Broda et al. 1995; Yoshida et al. 2004).

451 Although being accurate in quantitation of gene expression by detection of transcript abundances,
452 transcriptional analyses are seldom fully in accordance with protein production or enzyme activity
453 measurements due to for example mRNA half-life characteristics, post-transcriptional and post-
454 translational regulation processes, and gene family redundancy effects (Vogel and Marcotte 2012). For
455 example, transcript abundances of fungal *mnp* genes may not correlate with extracellular MnP activities
456 probably due to post-transcriptional or even post-translational processes affecting protein synthesis and
457 enzyme secretion (Kamei et al. 2008; Mancilla et al. 2010). We selected one or a few genes to represent
458 each main cellulolytic, hemicellulolytic and lignin-modifying activity of the corresponding CAZy classes in
459 our RT-qPCR study, based on our previous transcriptomic study of the fungus cultivated on solid wood.
460 For example, there are four unique GH7 cellobiohydrolase encoding genes identified in the *P. radiata*
461 genome, while the protein product of one gene (*cbhI_1*, target of this study) was the dominant CBH
462 enzyme detected on spruce wood (Kuuskeri et al. 2016). Differential regulation of multiple genes of each
463 CAZy class encoding similar and even overlapping activities, however, cannot be completely ruled out,
464 when the fungus is growing on a chemically and structurally dissimilar lignocellulosic substrate.

465 Our results imply that while growing on and bio-converting the solid lignocellulose core board
466 substrate under semi-aerobic conditions, simultaneous production of released sugars (saccharification)
467 and fermentation to ethanol by *P. radiata* was combined with significant up-regulation of expression of
468 the cellulolytic and hemicellulolytic CAZy genes. At the same time, genes encoding lignin peroxidases and
469 manganese peroxidases together with an apparently intracellular alcohol oxidase encoding gene
470 demonstrated differential expression according to the substrate and cultivation time-point. Thus,
471 expression of fungal oxidoreductase encoding genes seemingly is controlled by versatile regulatory events
472 and effectors, and not only by the lignocellulose substrate. Enzyme activity measurements confirmed
473 expression of the lignocellulose-degradation machinery of *P. radiata*, which has most likely originally
474 evolved for extraction of carbon from wood as the natural habitation environment for the fungus. The
475 production of wood-decay enzymes actively aided degradation, saccharification and utilization of the
476 processed and recycled fiber-based lignocellulose core board waste material.

477

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480

481 **Compliance with ethical standards**

482

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Author´s contributions

MM, HM and TL, designed the study. NR and MM carried out the experiments. MM, NR and HM analyzed the data. MM, HM and TL, interpreted the data. MM and TL wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Transcriptome data from the solid-state spruce wood and liquid malt extract cultivations of *P. radiata* including raw counts, log2 fold changes and *P* values for the genes selected for the RT-qPCR analysis. The gene data are derived from our previous study (Kuuskeri et al. 2016).

Gene ID (strand and locus ID in genome assembly)	Gene name	Counts Spruce-2w[1]	Counts Spruce-4w[2]	Counts ME[3]	log2FC[4] ME vs Spruce-2w	*P* value ME vs Spruce-2w	log2FC ME vs Spruce-4w	*P* value ME vs Spruce-4w	log2FC Spruce-2w vs Spruce-4w	*P* value Spruce-2w vs Spruce-4w
minus.g11349	*aox1*	121077.67	80700.00	100.00	11.15	0.00	9.60	8.9×10^{-260}	-1.56	1.5×10^{-7}
minus.g7505	*bgl1*	10849.33	2565.50	280.00	5.95	1.4×10^{-33}	3.00	8.5×10^{-8}	-2.95	8.6×10^{-7}
minus.g5595	*cbhI_1*	122080.33	146060.50	103.00	10.61	3.1×10^{-92}	9.83	3.2×10^{-65}	-0.78	0.30
plus.g7451	*eg1*	24871.33	52707.00	172.67	7.79	4.7×10^{-60}	7.84	1.0×10^{-49}	0.04	1.00
plus.g7011	*lacc1*	3996.00	9634.00	9640.67	ns[5]	ns	ns	ns	ns	ns
minus.g6827	*lip2*	237229.00	5283.50	63.33	12.65	0.00	6.44	4.9×10^{-91}	-6.21	2.9×10^{-92}
plus.g11059	*lip3*	807969.67	132487.50	874.33	10.72	0.00	7.30	5.8×10^{-116}	-3.41	5.9×10^{-25}
plus.g9320	*lpmo1*	83723.33	89681.00	75.33	10.77	4.0×10^{-233}	10.04	5.8×10^{-166}	-0.73	0.10
plus.g1419	*mnp1-long*	505031.33	315044.50	243.67	11.85	0.00	10.23	2.9×10^{-239}	-1.62	2.5×10^{-6}
plus.g10562	*mnp2-long*	71850.33	109034.00	4304.67	5.04	3.5×10^{-73}	4.66	1.5×10^{-50}	-0.38	0.40
plus.g453	*mnp3-short*	8506.67	4524.50	541.00	4.91	5.6×10^{-77}	3.14	5.3×10^{-26}	-1.77	3.7×10^{-8}
plus.g3697	*xyn1*	16377.00	7461.50	90.67	8.15	2.6×10^{-85}	6.15	4.8×10^{-40}	-2.00	1.2×10^{-4}

[1] two-week cultivation time point; [2] four-week cultivation time point; [3] malt extract; [4] fold change; [5] statistically not significant

743 **Table 2.** Primers designed for and used in the RT-qPCR analyses

Primer	Sequence 5´- 3´	Amplicon size (bp)	Amplification efficiency (%)	R^2 value	Reference[1]
Sar1F1	GACATTTGCAAGCTCGTCGG	181	92.6	0.997	This study
Sar1R1	TGCGTCGATCTTGTTACCGAG	181	92.6	0.997	This study
Ubc6F1	CACAAGCGACTGACCAAGGA	158	91.6	0.996	This study
Ubc6R1	CATTAGTACGCCGTGGTACTCTC	158	91.6	0.996	This study
Fis1F2	GCTCGGGCACTATAAGATGGG	137	100.4	0.997	This study
Fis1R2	ATATAGCCCTCTTGGGTGACCT	137	100.4	0.997	This study
PrLacc1qPCRF	GTTTCTCTCGCCAGGCTGT	114	76.1	0.991	This study
PrLacc1comp	TAGTTGCGTTGGTCAATTCG	114	76.1	0.991	Mäkelä et al. 2006
LiP2F1	GACCATTTGTTGGCTCGTGAC	106	96.1	1	This study
LiP2R1	CCCATAGCACCGAAGATAAACTG	106	96.1	1	This study
Pr79_LiP3_qPCR_F	CTCTGACGTCCTCCCCGT	79	74.1	0.999	This study
Pr79_LiP3_qPCR_R	CTCAACATCGTTGATCGTGATT	79	74.1	0.999	This study
MnP1-longF1	CTCCATCCTTGAACGCTTTGAG	139	96	0.997	This study
MnP1-longR1	TGAAAGGCGTGCTGTCGA	139	96	0.997	This study
PrMnP2FqPCR	GTTCCCCACGCTGTCCAC	133	90.1	0.996	This study
MnP2rev	CTACGAGTCGTCTGCACCAC	133	90.1	0.996	Hildén et al. 2005
PrMnP3F	ACCAGGGCGAGGTTGAGT	125	91.3	0.983	Mäkelä et al. 2006
PrMnP3R qPCR	CTGGATCTTGGCCTGGTTGT	125	91.3	0.983	This study
AOX1F1	CACAGCGATATCAAGTACACGA	200	92.8	1	This study
AOX1R1	CAAGTTATCCGGGCAGATACTG	200	92.8	1	This study
LPMO_AA9.1F2	GCTGAAGTCATTGCTCTCCAC	171	98.4	0.998	This study
LPMO_AA9.1R2	ACGACTGGTAAATGTTGATGAGG	171	98.4	0.998	This study
BGL_GH3.1F1	ACCACTGTAACTACATCGCTC	113	97.6	0.999	This study
BGL_GH3.1R1	GATATAACCCTCACCGCTATCTG	113	97.6	0.999	This study
EG_GH5.3F1	CGTGCTTACTACCCTCGTG	108	96.1	0.999	This study
EG_GH5.3R1	GGAGTTCAAGAATTGTTCGCAG	108	96.1	0.999	This study
CBH_GH7.1F1	CCGTCGCTCAGTATGGTCAG	102	101.9	0.998	This study
CBH_GH7.1R1	CACTGGGAGTAGTAAGGGTTGAC	102	101.9	0.998	This study
XYN_GH11.1F1	TCATCCGGCACAGTTGCT	112	96.3	1	This study
XYN_GH11.1R1	CATTGGCAGTAGTAGGTGTTGG	112	96.3	1	This study

744 [1]This study, if not otherwise mentioned

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750 **Table 3.** Functional annotation and characteristics of the *P. radiata* proteins encoded by the studied
751 genes. Closest homologues were searched by Blastp using megablast algorithm against the NCBI non-
752 redundant database.

Protein	CAZy family	Gene locus id	Functional annotation	Signal sequence[1]	Length (aa)	Carbohydrate-binding domain[2]	Closest homologue[3]	Reference
SAR1		plus.g4563	GTP-binding protein	-	190	-	OSX65412	This study
UBC6		minus.g11916	Ubiquitin conjugating enzyme	-	274	-	KIP03433	This study
FIS1		minus.g12021	Mitochondrial membrane fission protein	-	156	-	OKY59824	This study
LACC1	AA1	plus.g7011	Laccase	+	521	-	Q01679	Saloheimo et al. 1991; Mäkelä et al. 2006
LiP2	AA2	minus.g6827	Lignin peroxidase	+	371	-	AAW59419	Kuuskeri et al. 2016
LiP3	AA2	plus.g11059	Lignin peroxidase	+	362	-	Q53WT9	Lundell et al. 1993; Hildén et al. 2006
MnP1-long	AA2	plus.g1419	Manganese peroxidase	+	390	-	BAP05606	Kuuskeri et al. 2016
MnP2-long	AA2	plus.g10562	Manganese peroxidase	+	391	-	Q70LM3	Hildén et al. 2005
MnP3-short	AA2	plus.g453	Manganese peroxidase	+	363	-	Q96TS6	Hildén et al. 2005
AOX1	AA3_3	minus.g11349	Alcohol oxidase	-	651	-	CDG66232	This study
LPMO1	AA9	plus.g9320	Lytic polysaccharide monooxygenase	+	337	CBM1	OKY63746	This study
BGL1	GH3	minus.g7505	β-glucosidase	+	815	CBM1	AAC26490	This study
EG1	GH5_5	plus.g7451	β-1,4-Endoglucanase	+	389	CBM1	KIP12070	This study
CBHI_1	GH7	minus.g5595	Cellobiohydrolase	+	518	CBM1	BAA76363	This study
XYN1	GH11	plus.g3697	β-1,4-Endoxylanase	+	290	CBM1	KIP12198	This study

753 [1]N-terminal signal peptide identified (+), no signal peptide (-); [2]if not identified (-); [3] GenBank/UniProtKB/SwissProt
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Figure legends

Fig. 1 Relative fold changes of the expression of selected lignocellulose-degradation associated genes in solid-state waste lignocellulose core board cultures after two and four weeks of growth as compared with liquid medium cultures with glucose as the carbon source. Transcript abundances were normalized against the transcript signals of *sar1*, *fis1* and *ubc6*. Mean value of three biological replicate cultures with standard deviation (error bars) are shown. *Statistically significant difference between core board and glucose cultivations ($P < 0.01$, *t*-test).

Fig. 2 RT-qPCR analysis for selected lignocellulose-degradation associated genes in solid-state waste lignocellulose core board cultures after two and four weeks of growth. Transcript abundances were normalized against the transcript signals of *sar1*, *fis1* and *ubc6* genes. Mean value of three biological replicate cultures with standard deviation (error bars) are shown. *Statistically significant difference between the two time points ($P < 0.01$, *t*-test).

Fig. 3 RT-qPCR analysis for selected lignocellulose-degradation associated genes in liquid medium cultures on glucose after two and four weeks of growth. Transcript abundances were normalized against the transcript signals of *sar1*, *fis1* and *ubc6* genes. Mean value of three biological replicate cultures with standard deviation (error bars) are shown. * Statistically significant difference between the two time points ($P < 0.01$, *t*-test).

Fig. 4 Production of ethanol (a) by *P. radiata* and amount of reducing sugars (b) present in the core board and glucose cultivations at specific time points. Mean value of three biological replicate cultures with standard deviation (error bars) are shown.

Fig. 5 Production of manganese peroxidase (a) and laccase (b) activities by *P. radiata* during six weeks of core board and glucose cultivations. Mean value of three biological replicate cultures with standard deviation (error bars) are shown.

Fig. 6 Production of xylanase (a), endoglucanase (b), β-glucosidase (c) and cellobiohydrolase (d) activities by *P. radiata* during six weeks of core board and glucose cultivations. Mean value of three biological replicate cultures with standard deviation (error bars) are shown.